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Inhibitors and Promoters of Thalamic Neuron Adhesion and Outgrowth in Embryonic Neocortex: Functional Association with Chondroitin Sulfate

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Summary

When embryonic thalamic neurons are plated onto living slices of mouse forebrain, cell attachment and neurite outgrowth on different layers of the developing cerebral cortex vary dramatically, in ways that correlate with the timing and pattern of thalamocortical innervation. These layer-specific differences can be eliminated from embryonic day 16 slices by enzymatic removal of chondroitin sulfate (CS). The cortical plate (a zone avoided by thalamic axons in vivo) possesses inhibitory activity (anti-adhesive, neurite repelling) and the intermediate zone and subplate (in which thalamic axons normally grow) possess stimulatory activity (adhesive, neurite promoting), both of which are chondroitinase sensitive. These opposing activities appear not to reflect the presence of different CS proteoglycans (CSPGs) in different zones, but rather the presence of differentially localized CS-binding molecules, which can be competed away by soluble CS. This model reconciles conflicting reports on the actions of CSPGs in neural development, and suggests a role for CSPGs in the organization of matrix-bound cues in the brain.

Introduction

Extracellular matrix (ECM) molecules that have dramatic effects on neuronal adhesion, shape, migration, and neurite outgrowth are expressed in many regions of the developing nervous system, and are thought to play crucial roles in neural development (Sanes, 1989; Lander and Calof, 1993; Letourneau et al., 1994). Although its organization is not well understood, the ECM of the vertebrate CNS is unique in its relative lack of fibril- and polymer-forming molecules (collagens, elastin, laminins, etc.) and abundance of chondroitin sulfate proteoglycans (CSPGs) and hyaluronan (Margolis et al., 1975a; Toole, 1976; Lander and Calof, 1993).

CSPGs are a heterogeneous set of proteins that are substituted with glycosaminoglycans of the chondroitin sulfate (CS) class. A linear polymer of repeating disaccharides, CS is itself heterogeneous owing to variations in length, sulfation, and epimerization (the latter change producing structures known as dermatan sulfate). During development, strong immunostaining for CS often localizes to territories thought to act as barriers to migrating neurons or extending axons. These include the posterior sclerotome (Perris et al., 1991; Oakley and Tosney, 1991), the dorsal midline of the spinal cord and optic tectum (Oakley and Tosney, 1991; Hoffman et al., 1994, Soc. Neurosci., abstract), regions of developing retina (Snow et al., 1991; Brittis et al., 1992), and the epidermis and basal lamina of innervated skin (Kitamura, 1987). In vitro, CSPGs (Perris and Johansson, 1987; Snow et al., 1990; Friedlander et al., 1994; Dou and Levine, 1994; Maeda and Noda, 1996), the isolated core proteins of CSPGs (Dou and Levine, 1994; Maeda and Noda, 1996), and CS by itself (Carbonetto et al., 1983; Verna et al., 1989) have been shown to inhibit cell migration or neurite outgrowth on defined growth-promoting substrata. Data from organotypic cultures also suggest that inhibition of CSPG expression can allow neuronal migration or outgrowth into previously avoided territories (e.g., Fichard et al., 1991; Brittis et al., 1992).

Despite these data, it is clear that tissues that strongly express CS do not always exclude the entry of axons (Yaginuma and Oppenheim, 1991; Oakley and Tosney, 1991), and in some cases, CS immunostaining coincides with developing axon pathways (Sheppard et al., 1991; Bicknese et al., 1994; McAdams and McLoon, 1995). Paralleling these findings, several in vitro studies suggest that CSPGs (Streit et al., 1993; Faissner et al., 1994), CS (Lafont et al., 1992; Fernaud-Espinosa et al., 1994), and isolated core proteins (Iijma et al., 1991) promote rather than inhibit neurite outgrowth.

To understand better what role, if any, CSPGs play in axonal targeting, we chose to study the thalamocortical system. When thalamic axons enter the cerebral wall, they elongate within the subplate, a zone that immunostains strongly for CS (Bicknese et al., 1994; Miller et al., 1995). Although they encounter and even probe the overlying cortical plate, thalamic growth cones initially do not penetrate that zone (Lund and Mustari, 1977; Crandall and Caviness, 1984; Catalano et al., 1996). Eventually, once appropriate target neurons appear within the cortical plate, thalamic axons extend to those target cells. At about the same time, staining for CS in the cortical plate increases (Miller et al., 1995; Tuttle et al., 1995). Thus, the thalamocortical system provides examples of territories that axons both choose and avoid, as well as a changing landscape of CS expression over the course of development.

Recently, we showed that some of the cues to which thalamic axons respond in vivo can be rapidly detected in vitro by thalamic neurons cultured on vibratome slices of forebrain (Emerling and Lander, 1994). In the present study, we demonstrate that, in the developing cortex, at least two types of cues—permissive and repulsive—are responsible for the layer-specific behaviors of thalamic cells in this assay. We further show that both types of cues are functionally associated with CS, i.e., they require CS either for their biological activity or for their localization.

Results

Treatment of Embryonic Forebrain Slices with Chondroitinase ABC Alters Specific Attachment of Thalamic Neurons to Cortical Layers

Within 3 hr of plating onto living slices of pre- and postnatal mouse forebrain, embryonic thalamic neurons dis-

play layer-specific attachment and neurite outgrowth (Emerling and Lander, 1994): zones of the cerebral wall that thalamic axons enter in vivo (subplate and intermediate zone) support attachment of thalamic neurons in vitro, as do zones that thalamic axons do not normally enter (marginal and ventricular zones). In contrast, neurons attach poorly to the embryonic cortical plate, a zone that marks a boundary to thalamic axon growth in vivo. Furthermore, neurites extending in vitro from cells attached to the embryonic subplate and intermediate zone orient parallel with the cortical plate/subplate boundary and fail to enter the cortical plate (thalamic axons cross readily between other zones). When thalamic neurons are plated onto postnatal slices—a time when thalamic axons have invaded the lower layers of the former cortical plate (cortical laminae IV–VI)—they attach well to those layers that thalamic axons have invaded, but still attach poorly to higher (supragranular) cortical plate–derived layers (laminae II–III) (Emerling and Lander, 1994).

To examine whether CSPGs play a role in these layer-specific cell behaviors, embryonic day 14 (E14) thalamic neurons were cultured on E16 forebrain slices in the presence of chondroitinase ABC (Ch'ase), an endoglycoeliminase that degrades all forms of CS (including those containing dermatan sulfate sequences). Slices were incubated in Ch'ase (1 U/ml) or carrier (complete, serum-free medium) for 4 hr prior to the addition of thalamic cells. After 3 hr of further culture in the continued presence of either enzyme or carrier, slices were rinsed to remove unattached cells, fixed, and counterstained with bisbenzamide.

In cultures not exposed to enzyme, thalamic neurons attached in the expected nonuniform manner: they bound well to the subplate, intermediate zone, and marginal zone, somewhat less to the ventricular zone, and were almost absent from the cortical plate (Figure 1A). In contrast, when Ch'ase was included in the culture medium, attachment to the cortical plate was markedly enhanced and attachment to the subplate and intermediate zone was diminished. There was little change in attachment to the marginal and ventricular zones (Figure 1B). Levels of cell attachment to each layer were similar throughout the rostrocaudal extent of the neocortex in both treated and untreated cocultures, and layer-specific differences in cell distribution appeared only after cocultures were rinsed to remove nonattached cells (i.e., effects were not due to cell migration or differential settling of cells).

In the above experiment, Ch'ase presumably digested CS both in the forebrain slice and on the thalamic cells. To identify the required site of enzyme action, cultures were prepared in which forebrain slices were pretreated with Ch'ase, washed extensively, and cocultured with untreated thalamic cells, while a parallel set of cultures used Ch'ase-pretreated thalamic cells and untreated forebrain slices. In both cases, cycloheximide was included both during and after Ch'ase digestion to block resynthesis and replenishment of CSPGs. Controls were also performed in which cycloheximide alone was added. Cultures were rinsed, fixed, and counterstained, and the densities of labeled cells attached to specific zones determined for several cultures (Figure 2). As previously, data from the subplate and intermediate zone were combined, because the border between these zones could not be accurately determined (Emerling and Lander, 1994).

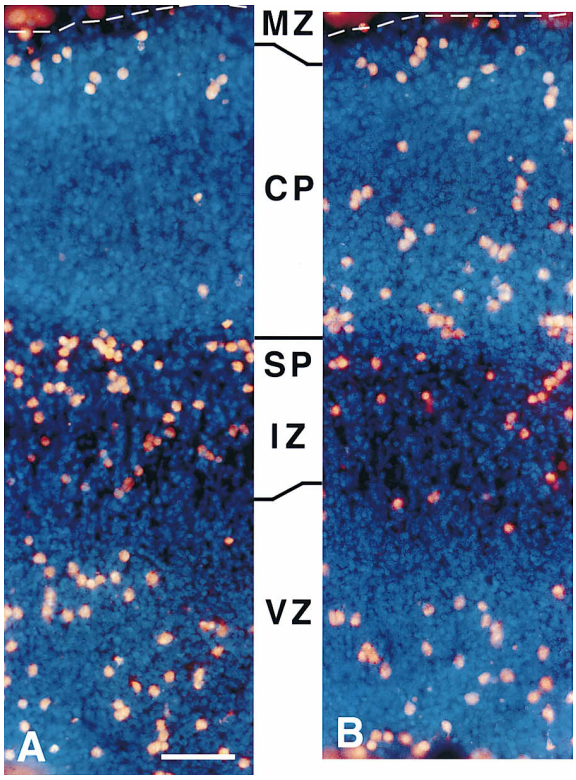

Figure 1. Effect of Ch'ase on Attachment of Thalamic Cells to E16 Cerebral Wall

Sagittal forebrain sections were exposed to culture medium (A) or medium containing 1 U/ml Ch'ase (B) for 4 hr at 37°C. Fluorescently labeled E14 thalamic neurons were plated onto the sections in the continued presence of either culture medium or Ch'ase for a further 3 hr. Slices were rinsed, fixed, and counterstained with bisbenzamide. The photos show corresponding regions of dorsomedial cerebral cortex. The nuclei of endogenous cells appear blue, and attached embryonic thalamic cells appear red. Zones can be discerned as differences in nuclear density. Broken white lines delineate the pial edge of the tissue. In (A), few cells have attached to the cortical plate (CP) while more are seen on the subplate/intermediate zone (SP/IZ), marginal zone (MZ), and ventricular zone (VZ). The addition of Ch'ase to the culture medium (B) caused more cells to attach to the cortical plate and fewer cells to attach to the subplate/intermediate zone. Bar, 50 μm.

In untreated cultures ("Cont"), or cultures treated only with cycloheximide ("CHX"), marked layer-specific differences in thalamic cell attachment, such as poor attachment of cells to the cortical plate, were easily measured. Ch'ase treatment ("Ch'ase + CHX") of thalamic cells did not alter these differences (Figure 2A), whereas Ch'ase treatment of forebrain slices had two effects (Figure 2B): it significantly increased thalamic cell attachment to the cortical plate *and* decreased attachment to the subplate/intermediate zone. Attachment to other zones was unaffected. These data imply that Ch'ase acts on forebrain tissue to cause changes in thalamic cell attachment.

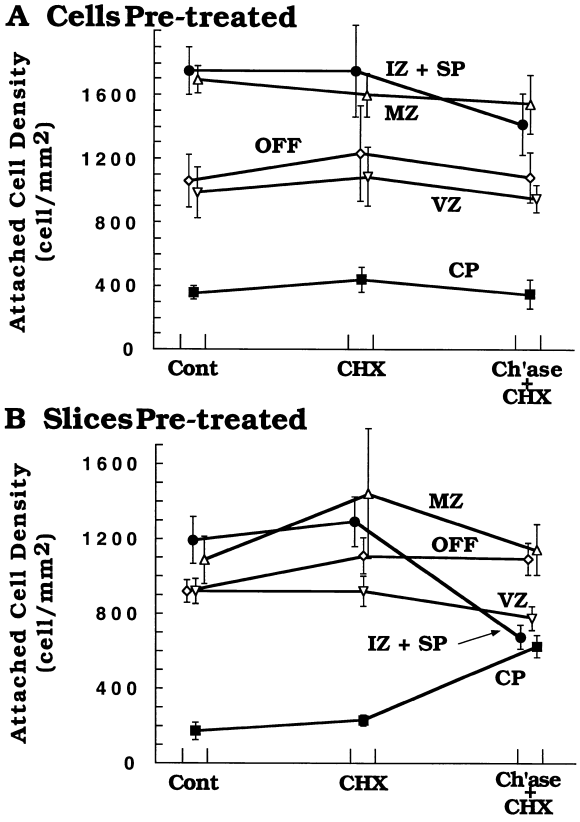

Figure 2. Cell Attachment after Ch'ase Treatment of Either Thalamic Cells or Forebrain Slices Alone

Thalamic neurons (A), or forebrain slices (B), were treated with balanced salt solution (Cont), cycloheximide (6 μg/ml, CHX), or 1 U/ml Ch'ase plus cycloheximide (Ch'ase + CHX), before being extensively rinsed and cocultured with untreated slices or cells, respectively (see Experimental Procedures). In both (A) and (B), coculture medium continued to contain cycloheximide (for CHX and Ch'ase + CHX) but did not contain Ch'ase. Mean densities (± SE) of cell attachment to the marginal zone (MZ, open triangle), the cortical plate (CP, closed square), the subplate/intermediate zone (IZ + SP, closed circle), and the ventricular zone (VZ, inverted open triangle) were calculated from eight (A) or seven (B) slices for each treatment. "Off slice" data (OFF, open diamond) were collected from the culture substratum adjacent to the pial surface. When thalamic cells alone were pretreated (A), neither cycloheximide nor Ch'ase significantly altered attachment to any zone (analysis of variance: $P_{all\ zones} > 0.2$). Treatment of slices with Ch'ase (B), however, did alter cell attachment to the cortical plate ($P_{cp} < 0.001$) and the subplate/intermediate zone ($P_{sp/iz} < 0.005$) while not significantly affecting attachment to other zones ($P_{mz} > 0.2$, $P_{vz} > 0.2$, $P_{off\ slice} > 0.2$). Mean attachment to the cortical plate increased about 4-fold, whereas attachment to the subplate/intermediate zone decreased by ~50%. Newman-Keuls analysis verified that both effects were caused by the combined Ch'ase/cycloheximide treatment (Ch'ase + CHX) and not by cycloheximide alone (CHX): $\mu_{cont,cp} = \mu_{chx,cp} \neq \mu_{ch'ase\ +\ chx,cp}$ and $\mu_{cont,sp/iz} = \mu_{chx,sp/iz} \neq \mu_{ch'ase\ +\ chx,sp/iz}$.

The Effects of Chondroitinase Correlate with the Removal of CS

The effects of Ch'ase on thalamic cell attachment were dose dependent (Figure 3), with significant increases in attachment to the cortical plate and decreases in attachment to the subplate/intermediate zone seen at enzyme concentrations in the range used to digest CS from trace amounts of proteoglycans (PGs) in solution (0.02–0.2 U/ml; cf. Herndon and Lander, 1990). For both the cortical plate and the subplate/intermediate zone, mean cell attachment at 0.2 U/ml and 2.0 U/ml enzyme were not significantly different and, compared with control levels, correspond to a ~5-fold increase in attachment to the cortical plate and a ~50% decrease in attachment to the subplate/intermediate zone.

To test whether the dose dependence of these effects correlated with the removal of CS from the slice, slices were treated with enzyme as in Figure 3 and immunostained with CS-56, an antibody that recognizes both the 4- and 6-sulfated forms of CS (Avnur and Geiger, 1984). In slices not exposed to enzyme (Figure 4A and 4B), CS immunoreactivity corresponded with that reported in vivo: the marginal zone and subplate regions stain more densely than other zones (Sheppard et al., 1991; Tuttle et al., 1995; Miller et al., 1995; unpublished data). After treatment with Ch'ase (Figures 4C and 4D), overall staining decreased and differences among zones were less noticeable. Immunofluorescence was quantified in both the cortical plate and the subplate/intermediate zone of slices treated with different Ch'ase concentrations (Figure 5). For both regions, there was a noticeable decrease in CS immunoreactivity after exposure to 0.02 U/ml Ch'ase, and a significant decrease across the dose curve. Overall, the concentration range over which CS immunoreactivity was removed from the slice paralleled the range over which alterations in cell attachment occur.

Although these data support the idea that digestion of CS causes changes in cell attachment, the possibility remains that the effects are produced by another enzymatic activity of Ch'ase, the ability to degrade the glycosaminoglycan hyaluronan (Yamagata et al., 1968), which is abundant in the developing brain (Margolis et al., 1975a; Toole, 1976). To test this possibility, a lyase specific for hyaluronan (streptomyces hyaluronidase) was added to cocultures. At no hyaluronidase concentration were effects on thalamic cell attachment to any zone seen (Figure 6). Staining with a biotin-conjugated hyaluronan-binding protein verified that most of the hyaluronan was removed at the enzyme concentrations used (data not shown). Use of even greater hyaluronidase concentrations (500 TRU/ml) caused forebrain slices to disintegrate (data not shown), an effect never seen with Ch'ase.

Chondroitinase Treatment Alters the Relative Permissiveness of Subplate and Cortical Plate Tissue for Thalamic Neurite Outgrowth

Previously (Emerling and Lander, 1994), we proposed that layer-specific differences in attachment of thalamic neurons to embryonic cortex reflected the presence of localized axon guidance cues, since similar layer-specific effects were seen in the behavior of neurites extended by attached thalamic neurons. In particular, neurites that originated on the subplate/intermediate zone rarely crossed into the cortical plate, even when they originated within one cell diameter of the border between the two zones.

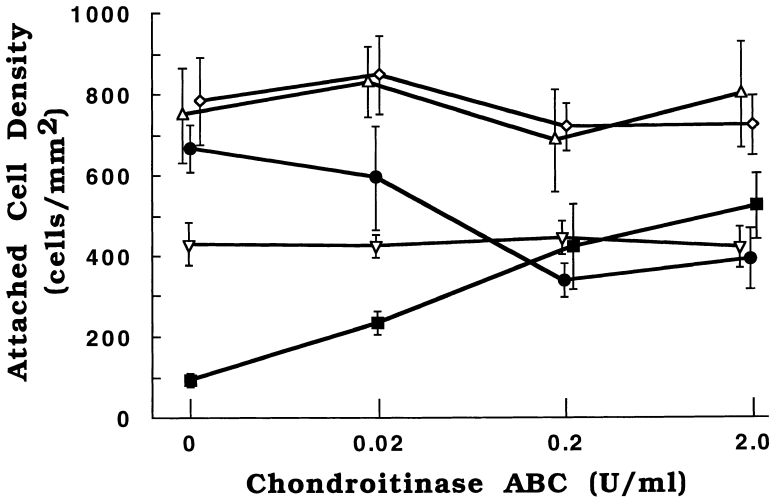

Figure 3. Effects of Ch'ase Dose on Thalamic Cell Attachment

Forebrain slices were exposed to various concentrations of Ch'ase for 4 hr, followed by coculture with embryonic thalamic neurons in the continued presence of Ch'ase (at the same concentration). After 3 hr, slices were rinsed, fixed, and counterstained. Densities of cells attached to the marginal zone (open triangle), the cortical plate (closed square), the subplate/intermediate zone (closed circle), and the ventricular zone (inverted open triangle), as well as to the culture substratum ("off slice,"), were determined from eight cultures for each dose. Mean values (± SE) are shown, and demonstrate the effect of Ch'ase on attachment to the cortical plate and the subplate/intermediate zone, but not on attachment to other zones (analysis of variance: $P_{cp} < 0.001$; $P_{sp/iz} < 0.03$; $P_{mz} > 0.2$; $P_{vz} > 0.2$; $P_{off\ slice} > 0.2$). Mean cell density on the cortical plate increased ~5-fold ($X_0 = 92$, $X_{2.0} = 522$ cell/mm^2), whereas attachment to the subplate/intermediate zone decreased about 50% ($X_0 = 666$, $X_{2.0} = 393$ cell/mm^2).

In the present study, we examined neurite outgrowth near the cortical plate border after Ch'ase treatment. Figures 7A and 7B show a typical neurite from an attached thalamic cell in a control culture (no enzyme); the neurite runs parallel to the subplate boundary and fails to enter the cortical plate. In contrast, in the presence of Ch'ase (1 U/ml), neurites readily cross from the subplate to the cortical plate (Figures 7C and 7D). Also visible after Ch'ase treatment are neurites that originate from the numerous cells that now attach to the cortical plate itself.

Results were quantified by scoring neurites originating within 25 μm of the border between the cortical plate and subplate as either having crossed or not crossed this border (Table 1). The proportion of all neurites that crossed was significantly increased by Ch'ase. A similar increase was seen for just those neurites that originated on the subplate. We could not establish whether there was also an increase in the proportion of neurites crossing from the cortical plate onto the subplate, since so few cells attach to the cortical plate in the absence of Ch'ase that the number of neurites originating there under control conditions is extremely low (Table 1). Treatment of similar cultures with streptomyces hyaluronidase produced no noticeable effects on neurite behavior (data not shown).

CS May Act Indirectly, by Immobilizing Other Molecules in the ECM

These results indicate that at least two activities in the embryonic cortex that influence thalamic neurons are functionally associated with CS: one activity is localized to the subplate/intermediate zone and promotes thalamic neuron attachment; another is localized to the cortical plate and inhibits attachment. One or both activities renders the cortical plate nonpermissive for the entry of axons from the underlying subplate.

If CSPGs are *directly* responsible for mediating these two opposing activities, it is reasonable to assume that different CSPGs would have to be involved in the different zones. Thus, we looked for CSPGs in E16 cerebral wall that are present in the cortical plate but not in the subplate/intermediate zone, or present in the subplate/intermediate zone but not in the cortical plate. PGs were isolated and radiolabeled (Herndon and Lander, 1990) from zones individually dissected from E16 slices, and analyzed by comparing core protein banding patterns before and after treatment with glycosaminoglycan lyases (Figure 8). The core proteins of several heparan sulfate–containing PGs, as well as some copurifying non-PGs, were found either exclusively or predominantly in certain zones. However, no CSPG (or mixed heparan sulfate/CSPG) core proteins were found exclusively or predominantly in the cortical plate or subplate/intermediate zone.

Although there may be differences in CSPG structure or expression that could not be detected by this analysis (or both) (see Discussion), the data raise the possibility that CS-dependent activities of the cortical plate and the subplate/intermediate zone may not reside in zone-specific CSPGs themselves, but rather in zone-specific molecules associated with CSPGs. The idea that biologically active proteins are held within the ECM by glycosaminoglycans is precedented (Roberts et al., 1988; Moscatelli, 1992), and proteins that bind glycosaminoglycans, including CS, are known to influence cell adhesion and neurite outgrowth (see Discussion). According to this view, removal of CS from cortical tissue alters thalamic neuron attachment and outgrowth by causing the release of CS-bound factors. If this is correct, one should also be able to release the same CS-bound factors by adding an excess of soluble CS, to displace them.

The experiment shown in Figure 9 tests this prediction. Forebrain slices were pretreated with CS (500 μg/ml) and extensively rinsed, prior to coculture in medium without added CS. Cycloheximide was included during pretreatment and coculture to prevent resynthesis of CSPGs and CS-binding proteins. CS pretreatment both

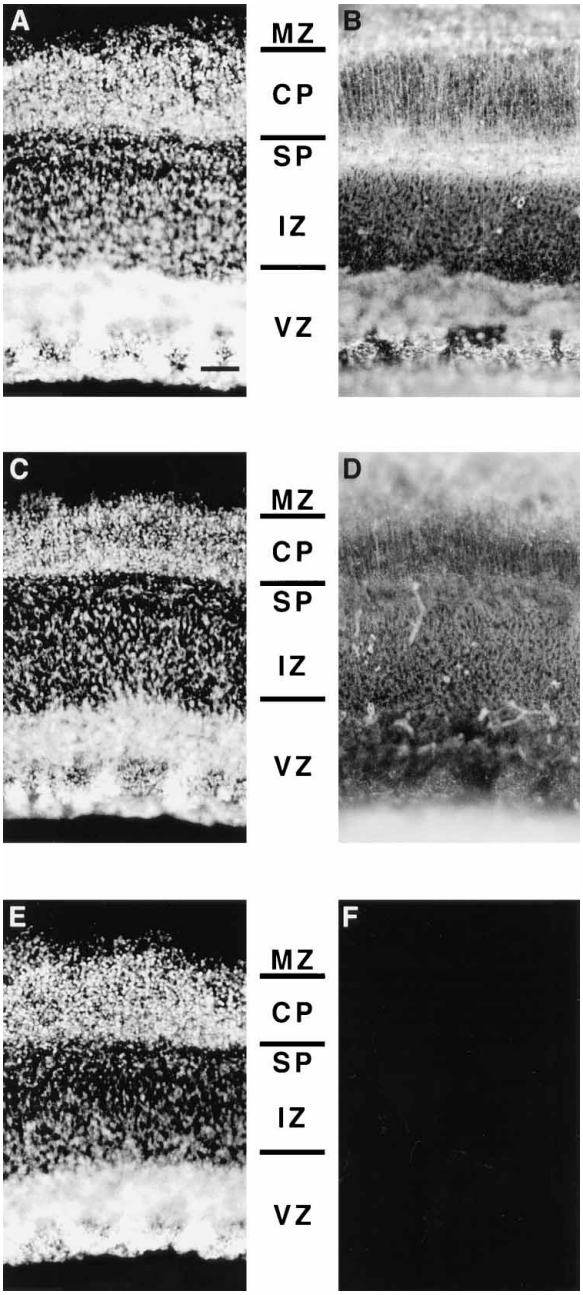

Figure 4. Effect of Ch'ase on CS Immunoreactivity

Sagittal forebrain slices were cultured at 37°C for 4 hr in the presence of either complete culture medium ([A and B] and [E and F]) or medium containing 2 U/ml Ch'ase (C and D). Slices were rinsed, fixed, incubated with the CS-56 antibody ([A and B] and [C and D]), or no first antibody (E and F), followed by a Cy3-conjugated secondary antibody and counterstained with bisbenzimide. Regions of dorsomedial cortex were photographed using bisbenzimide optics (A, C, and E) to reveal zones, and rhodamine optics (B, D, and F) to reveal bound secondary antibody. Photographic exposure times in (B), (D), and (F) were identical. In an untreated slice (A and B), the strongest CS immunoreactivity is seen in the subplate (SP) and marginal zone (MZ). Less intense staining is also seen in the intermediate zone (IZ), ventricular zone (VZ), and cortical plate (CP). Treatment with Ch'ase (C and D) lowered overall CS immunoreactivity and attenuated the zone-dependent pattern of immunostaining. Note that cell layers themselves were not disrupted by Ch'ase. A control slice (E and F) incubated with secondary antibody alone produced little fluorescence. Bar, 50 μm.

increased the level of attachment to the cortical plate and decreased attachment to the subplate/intermediate zone. Attachment to other zones, or to the culture substratum, remained essentially unchanged. Treatment of slices with CS did not alter the general pattern of CS immunoreactivity, although immunoreactivity was somewhat elevated in all zones (Figure 10), consistent with nonspecific binding or trapping of a small fraction of the added CS. Overall, the data strongly suggest that the displacement of CS-binding proteins from the cortical plate and subplate/intermediate zone accounts for the marked effects of Ch'ase on the ability of these zones to support cell attachment.

Discussion

The results presented here demonstrate that treatment of the embryonic cerebral wall with Ch'ase dramatically alters the layer-specific behaviors of thalamic neurons plated upon it. Enzyme treatment caused the embryonic cortical plate, normally a poor substratum for cell attachment and neurite outgrowth, to support attachment and outgrowth at levels comparable with those seen on other zones. Simultaneously, the subplate and intermediate zone, normally excellent substrata for attachment and outgrowth, came to support only half the level of attachment seen under control conditions (Figures 2 and 3). Paralleling these changes in cell attachment were changes in neurite outgrowth, such that thalamic neurites crossed frequently between the subplate and the cortical plate, a phenomenon rarely seen in control cultures (Figure 7; Table 1). Analysis of PG expression in different cortical zones failed to reveal any CSPGs with appropriate zone-specific expression to account for these Ch'ase-sensitive cell behaviors (Figure 8). However, similarities between the effects of treatment with Ch'ase and treatment with exogenous CS (Figure 9) suggested that molecules bound to CS are responsible for the observed zone-specific activities.

Adhesive and Repulsive Cues in the Developing Cortex

Previously (Emerling and Lander, 1994), we argued that layer-specific differences in thalamic attachment and neurite outgrowth on cortical tissue are likely to reflect axon guidance cues within the developing cortex. In that study, we were unable to resolve whether the poor attachment of thalamic cells to the embryonic cortical plate results from a lack of promoters of adhesion or the presence of inhibitors of adhesion. We were also unable to address whether any of the cues detected by thalamic neurons in forebrain slices were intrinsic to the cortical tissue, or associated with thalamic axons already present within the slice.

The present study resolves both these issues. The fact that treatment with Ch'ase (or CS) renders the cortical plate a good substratum both for attachment and neurite ingrowth indicates that one or more inhibitory (anti-adhesive, neurite-repellent) factors is found in the embryonic cortical plate. Similarly, the data also reveal a stimulatory (adhesive) activity in the subplate/interme-

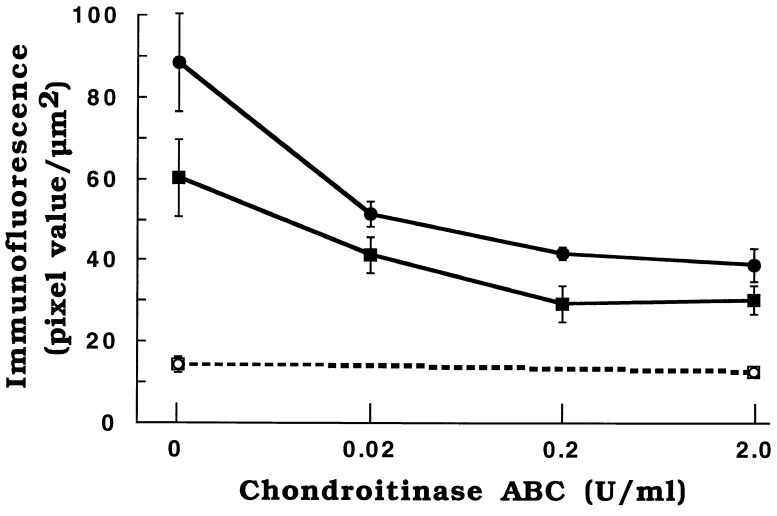

Figure 5. Dose-Dependent Removal of CS Immunoreactivity by Ch'ase

Forebrain slices were cultured in the presence of various concentrations of Ch'ase for 4 hr at 37°C and stained as in Figure 4. Digitized images were collected and fluorescence quantified for each zone (see Experimental Procedures). Data from the cortical plate (closed square) and subplate/intermediate zone (closed circle) represent mean values (± SE) for four slices from each dose and demonstrate that Ch'ase removes a substantial fraction of the CS immunoreactivity (analysis of variance: $P_{cp} < 0.02$; $P_{sp/iz} < 0.001$). To control for the possibility that Ch'ase affects tissue autofluorescence or nonspecific binding of the secondary antibody, untreated (n = 4) and Ch'ase treated (2 U/ml; n = 4) slices were incubated exclusively with secondary antibody and fluorescence levels determined for the cortical plate (open square) and the subplate/intermediate zone (open circle). Data are mean values (± SE), and were not altered by Ch'ase treatment (t test: $P_{cp} > 0.4$, $P_{sp/iz} > 0.5$). Differences between "background" fluorescence (dotted line) and fluorescence levels at saturating concentrations of Ch'ase presumably represent CS that was inaccessible to the lyase (e.g., CS "stubs" left at protein attachment sites, intracellular CS) or nonspecific primary antibody binding.

diate zone. Since the cortical plate does not contain endogenous thalamic axons (at this stage), at least the activity localized to this zone must be intrinsic to the cortex.

Together with our earlier results, these data imply that the cortical plate produces at least one neurite-repellent molecule at a time when thalamic axons normally contact it, but fail to enter it. It is likely that this molecule (or molecules required for expression of its inhibitory activity) is down-regulated as neuronal laminae differentiate from the cortical plate, since the in vitro nonpermissiveness of the lower cortical plate (laminae IV–VI) disappears (Emerling and Lander, 1994) at postnatal stages (i.e., after thalamic axons have invaded it). Although it is tempting to speculate that the persistence of nonpermissiveness in the remaining cortical plate–derived laminae (II–III) is due to the persistence of this molecule, we suspect this is not the case, since treatment of postnatal day 7 forebrain slices with Ch'ase failed to alter the nonpermissiveness of the supragranular laminae (unpublished data).

The idea that the embryonic cortical plate is inherently nonpermissive for thalamic axon growth and becomes more permissive postnatally was also proposed by Tuttle et al. (1995). They allowed thalamic neurites to choose between substratum-bound membranes isolated from postnatal or embryonic cortical plate. Although the embryonic cortical plate was a less preferred substratum for growth, the experiments failed to detect the presence in it of inhibitors of neurite growth. However, in preparing their membranes, CSPGs were shown to be lost (along with CSPG-bound proteins, presumably), so it is unlikely that Tuttle et al. would have detected the same inhibitory activity that we encountered in living tissue slices. Together, the data suggest that cues of different types, stimulatory and inhibitory, CS-dependent and CS-independent, act in concert to regulate the timing of thalamic axon invasion of the cortical plate.

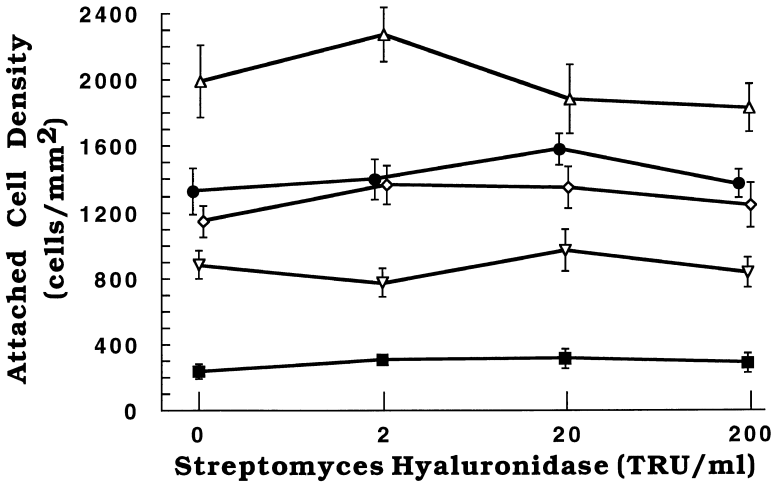

Figure 6. Lack of Effect of Hyaluronidase on Thalamic Cell Attachment

E16 forebrain slices were exposed to culture medium or streptomyces hyaluronidase for 4 hr at 37°C, before coculture with embryonic thalamic neurons in the continued presence of enzyme. After 3 hr, slices were rinsed, fixed, and counterstained. Densities of cells attached to the marginal zone (open triangle), the cortical plate (closed square), the subplate/intermediate zone (closed circle), and the ventricular zone (inverted open triangle), as well as to the culture substratum ("off slice," open diamond), were determined from eight cocultures for each dose. Mean values (± SE) are shown, and fail to demonstrate an effect of any dose on attachment to any zone (analysis of variance: $P_{all\ zones} > 0.2$).

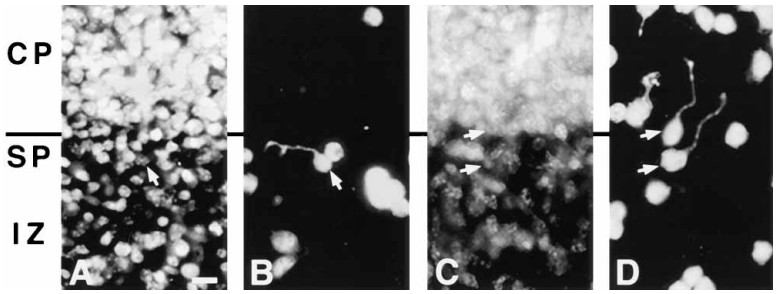

Figure 7. Thalamic Neurite Extension on the Cortical Plate and Subplate in Cocultures Treated with Ch'ase

Micrographs show the cortical plate (CP), subplate (SP), and intermediate zone (IZ) of bisbenzamide-stained cocultures treated with culture medium (A), or 1 U/ml Ch'ase (C) as in Figure 1. The same slices viewed under rhodamine optics reveal attached thalamic cells and their neurites (B and D). Arrows indicate neurite-bearing thalamic cells and their corresponding nuclei. In control cultures (A and B), neurites that grew on the subplate/intermediate zone tended to orient parallel with the zone borders. Neurites that crossed from the subplate onto the cortical plate were extremely rare, as were neurites that originated in the cortical plate (Emerling and Lander, 1994). However, when Ch'ase was present (C and D), neurite outgrowth on the cortical plate was enhanced and processes that originated on the subplate often crossed onto the cortical plate. Bar, 10 μm

Activities Associated with CS May Reflect the Functions of Proteins Bound to Proteoglycans

Numerous authors have argued that CS and CSPGs are inhibitory to axon growth, whereas others, using different assays, have argued that these molecules are growth promoting (see Introduction). The present study demonstrates that, within a single assay, CSPGs can be implicated in both kinds of effects. Interestingly, little correlation exists between levels of overall CS expression and either stimulatory or inhibitory activity. The embryonic cortical plate, for instance, is the site of relatively weak CS immunoreactivity, but potent CS-dependent inhibition of attachment and neurite outgrowth. Both the subplate and marginal zone stain strongly for CS, but only the former exhibits CS-dependent attachment activity.

Apparently, to understand the origins of the CS-dependent activities described in the present study, one must look beyond the bulk properties of CS. There are two possible explanations for the opposing CS-dependent activities in the cortical plate and the subplate/intermediate zone: these zones could contain CSPGs that have inherently different biological activities, or they could owe their differences to the activities of proteins that are not CSPGs, but whose functions are CS dependent (e.g., CS-bound proteins).

Although our analysis of PGs isolated from different zones failed to identify any CSPGs that were conspicuously present in, or absent from, the cortical plate or subplate/intermediate zone, these negative results do not prove that no such CSPGs exist. The conditions used for tissue extraction are known to solubilize most, but not all, of the CS in the brain (Margolis et al., 1975b). It is also possible that some CSPG core proteins were missed because they did not label well with ^{125}I; they were too poorly sulfated to bind to DEAE–cellulose, or they were too rare to be noticed. Indeed, others have reported that immunoreactivity for the CSPG neurocan is more intense in the subplate and marginal zone than in the cortical plate and ventricular zone (Miller et al., 1995; Meyer-Puttlitz et al., 1996) and cortical immunoreactivity for the CSPG phosphacan may (Meyer-Puttlitz et al., 1996) or may not (Miller et al., 1995) show zone-specific expression. Whether immunoreactivity reliably indicates the level of expression of these CSPGs remains to be seen, especially since the mRNA for these core proteins appears to localize mainly to zones different from those that immunostain strongly for them (Engel et al., 1996).

Even if there are no zone-specific CSPG core proteins in the cerebral wall, the possibility remains that differences in CS structure could assign different biological activities to common core proteins (cf. Faissner et al.,

Table 1. Neurite Crossing Between the Subplate and Cortical Plate

Neurites	Culture Medium	Crossed Border	Failed to Cross Border	Percentage Crossed
All neurites originating within 25 μm of subplate-cortical plate boundary	Control	6	203	3%
	Chondroitinase	80	219	27%
Neurites originating on cortical plate	Control	5	3	*
	Chondroitinase	38	148	20%
Neurites originating on subplate	Control	1	200	0.5%
	Chondroitinase	42	71	37%

Cocultures of embryonic thalamic cells and forebrain slices were exposed to culture medium (control) or 1 U/ml Ch'ase as in Figure 1. All neurites originating within 25 μm of either side of the subplate/cortical plate border were scored from 14 control and 24 treated cocultures. A neurite was defined as having crossed the border if at least half of the length of the neurite was on the opposite side from the point of origin of the neurite; other processes were labeled as having not crossed. The frequency of neurite crossings increased with enzyme treatment ($P < 0.001$, chi-squared analysis). Analysis of just those processes that originated on the subplate also shows that they cross onto the cortical plate much more often in Ch'ase treated cocultures ($P < 0.001$). Too few neurites originated on the cortical plate under control conditions to make statistical conclusions about the effects of enzyme on these processes (asterisk).

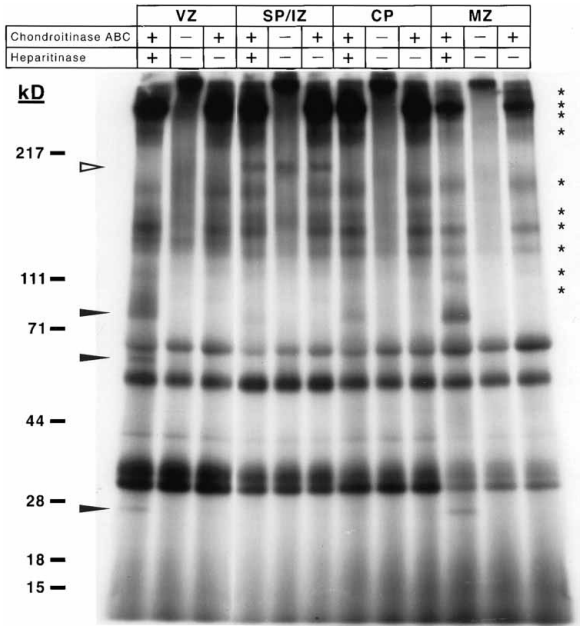

Figure 8. Analysis of PGs in Different Zones of the E16 Cerebral Wall

PGs were purified from microdissected zones and radioiodinated (see Experimental Procedures). Samples from the marginal zone (MZ), cortical plate (CP), subplate plus intermediate zone (SP/IZ), and ventricular zone (VZ) were treated with Ch'ase, Ch'ase plus heparitinase, or left untreated prior to separation by SDS–PAGE under reducing conditions. Bands that appear only after enzyme treatment represent the core proteins of PGs. Core proteins of heparan sulfate–containing PGs (filled arrowheads) that show zone-dependent expression include cores of ~26 kD (presumptive M16; Herndon and Lander, 1990) and ~88 kD (presumptive syndecan-1; Ivins and Lander, unpublished data) in the VZ and MZ and ~67 kD (presumptive glypican; Litwack et al., 1994) in the VZ. One ~206 kD non-PG (open arrowhead) is found exclusively in the SP/IZ, while another is seen exclusively in the VZ (~170 kD, not indicated). No CSPG or heparan sulfate/CS hybrid cores are exclusively expressed in the CP or SP/IZ. Several PG cores (asterisks at right), however, are seen in all zones (CSPGs at ~100 kD, ~137 kD, ~154 kD, ~168 kD, ~188 kD, and at least four > 217 kD), as well as a heparan sulfate-containing PG at ~118 kD.

1994). Yet despite these caveats, the results of one experiment in the present study independently suggests that CSPGs themselves are not directly responsible for zone-specific adhesive and repulsive activities. The fact that both the removal of CS (Ch'ase treatment) and the addition of excess soluble CS have the same effects on attachment to the cortical plate and the subplate/intermediate zone strongly suggests that both treatments work by disrupting CS-mediated binding of molecules that need not themselves be PGs. Intriguingly, these data parallel those of Silver and colleagues, who found that treatment of retinal explants with either Ch'ase (Brittis et al., 1992) or soluble CS (Brittis and Silver, 1994) caused ganglion cell processes to extend into a region (the ventricular surface) they would normally not enter. It may be that CS-bound molecules control axon growth in many regions of the nervous system.

This idea is exciting, because at least two families of ECM proteins—the tenascins and the thrombospondins—are known to bind CS or CSPGs, to be expressed in the developing brain, and to sometimes have anti-adhesive or neurite repellent effects (or both) in tissue culture (e.g., O'Shea and Dixit, 1988; Pesheva et al., 1989; Faissner and Kruse, 1990; Winnemöller et al., 1992; Grumet et al., 1994; Barnea et al., 1994). Tenascin-C, in particular, is often localized to regions of the CNS that act as boundaries to cell migration and neurite outgrowth (Faissner and Steindler, 1995), including the embryonic cortical plate (Tuttle et al., 1995). In addition to these ECM molecules, two other classes of secreted molecules that can provide repulsive cues to some types of axons bind glycosaminoglycans: semaphorins (collapsins) and netrins (Raper and Kapfhammer, 1990; Colamarino and Tessier-Lavigne, 1995). At least some of the semaphorins are expressed in mouse embryonic cortex (Püschel et al., 1996). However, only netrin-1 has so far been shown to bind specifically to CSPGs (Litwack et al., 1995, Soc. Neurosci., abstract).

Do CS Proteoglycans "Organize" the Brain Extracellular Matrix?

Is it coincidental that disruption of CS eliminates both stimulatory and inhibitory properties of cortical tissue, effectively erasing differences in the ability of different zones to selectively support thalamic attachment and restrict thalamic neurite growth? One explanation why a single treatment can have such dramatic effects may be that CSPGs play a general role in organizing the brain ECM, such that interference with the interactions of CS causes the removal or inactivation of a large number of ECM-associated molecules. A similar role has been proposed for heparan sulfate PGs in other types of ECMs, especially with regard to the sequestration of polypeptide growth factors (Roberts et al., 1988; Moscatelli, 1992; Klagsbrun, 1992). In the brain, however, CS is much more abundant than heparan sulfate (Margolis et al., 1975a), and most of the heparan sulfate PGs found in the brain so far are cell-surface, rather than ECM, molecules (Lander, 1993).

The glycosaminoglycan hyaluronan is also very abundant in brain and likely plays a major role in organizing the ECM (Margolis et al., 1975a; Toole, 1976; Bignami et al., 1993). However, experiments described here suggest an interesting functional difference between it and CSPGs: treatment of forebrain slices with high levels of hyaluronidase caused overt disintegration of the tissue, whereas Ch'ase did not. These results suggest a model in which hyaluronan plays a structural role in brain ECM, analogous perhaps to that of collagens in other ECMs. CSPGs might then associate with the matrix via hyaluronan (many CSPGs, including several in brain, bind hyaluronan via their core proteins [Zimmermann and Ruoslahti, 1989; Rauch et al., 1992; Iwata et al., 1993; Yamada et al., 1994]). Finally, other molecules that interact directly with cells would become incorporated into the ECM via interactions with the CS chains of CSPGs. It will be interesting to test the predications of this model, especially as they relate to the mechanisms of localization of numerous glycosaminoglycan-binding factors that are thought to influence neuronal proliferation, survival, cell migration, and axon guidance (Lander and Calof, 1993; Eckenstein, 1994; Shepherd et al., 1996).

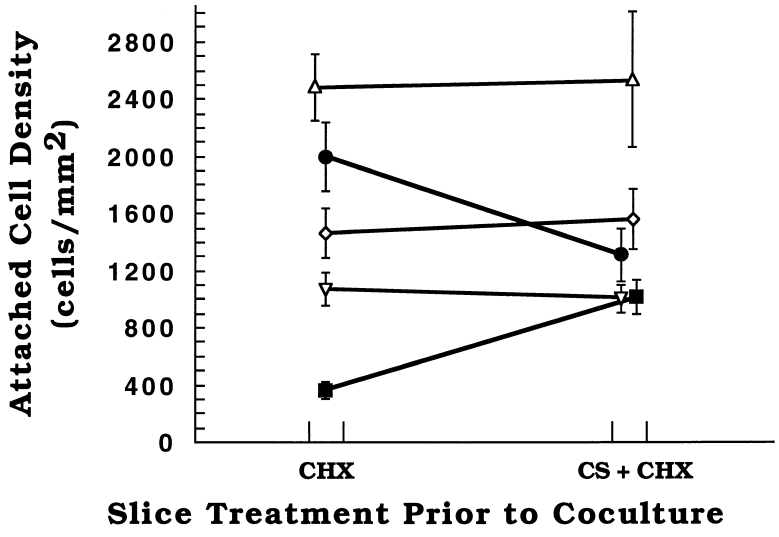

Figure 9. Thalamic Cell Attachment after Pretreatment of Slices with CS

E16 forebrain slices were treated with culture medium containing cycloheximide (6 μg/ml) and either lacking (CHX) or containing (CS + CHX) 500 μg/ml CS. Slices were extensively rinsed and cocultured with untreated cells in cycloheximide-containing medium (see Experimental Procedures). Mean densities (± SE) of cells attached to the marginal zone (open triangle), the cortical plate (closed square), the subplate/intermediate zone (closed circle), and the ventricular zone (inverted open triangle) were calculated from 23 cocultures ($n_{chx} = 11$, $n_{cs + chx} = 12$). "Off slice" data (open diamond) were collected from the culture substratum adjacent to the pial surface. Cycloheximide alone had no effect on cell attachment (see Figure 2). Cells attached well to the marginal zone, subplate/intermediate zone, and ventricular zone, but poorly to the cortical plate (analysis of variance: $P < 0.0005$; Newman-Keuls: $\mu_{chx,mz} = \mu_{chx,sp/iz} \neq \mu_{chx,vz} \neq \mu_{chx,cp}$). Pretreatment of slices with CS caused a ~3-fold increase in attachment to the cortical plate (t test: $P < 0.001$). Mean density of attachment to the subplate/intermediate zone also decreased (t test: $P = 0.03$). Attachment to other zones was not affected ($P_{mz} > 0.5$; $P_{vz} > 0.5$; $P_{off\ slice} > 0.5$). CS pretreatment caused levels of attachment to the cortical plate and subplate/intermediate zone to be comparable (analysis of variance: $P < 0.0005$; Newman-Keuls: $\mu_{cs + chx,mz} \neq \mu_{cs + chx,sp/iz} = \mu_{cs + chx,cp} = \mu_{cs + chx,vz}$).

Experimental Procedures

Preparation of Slice Cultures

Methods for slice coculture were as described (Emerling and Lander, 1994). In brief, E14 thalami were dissected from CD-1 mouse embryos, dissociated, and labeled with Cell Tracker™ (Molecular Probes, Eugene, OR) fluorescent dye. For pretreatment with Ch'ase, dissociated cells were agitated for 90 min at 37° in serum-free complete medium (CM, Emerling and Lander, 1994), CM with 6 μg/ml cycloheximide (Sigma), or CM with cycloheximide plus Ch'ase (Seikagaku; reconstituted with 5 mg/ml bovine serum albumin [BSA, crystaline grade, ICN] in calcium- and magnesium-free Hank's balanced salt solution, buffered with 10 mM HEPES (pH. 7.2) [HHBSS]). Cells were centrifuged through a cushion of 4% BSA in HHBSS and diluted to 1.8×10^6 cells/ml before plating.

E16 forebrains were embedded in low melting point agarose, sliced with a vibratome to 160 μm thick, collected on nitrocellulose filter disks (12 mm diameter; 2–4 slices/disks), and briefly stored in CM. Medium was replaced with 90 μl of fresh CM or fresh CM containing one or more of the following: Ch'ase; streptomyces hyaluronidase (Seikagaku); cycloheximide; or a mixture of CS isomers (4:1 ratio of CS A from bovine trachea and CS B from bovine mucosa [Sigma]) reconstituted to 500 μg/ml total in CM. The CS mixture, which contained some of chondroitin 4-, chondroitin 6-, and dermatan sulfate, was chosen so that the final concentrations of each of the different CS isomers were, respectively, ~30-fold, ~10-fold, and ~10-fold greater than the dissociation constant ($K_d = 490$ nM) for the binding of CS (from bovine trachea) to thrombospondin-1 (Herndon, 1996). Slices were kept at 37°C in a humidified 8% CO_2 atmosphere for 4 hr before being rinsed 3 times in ~14 ml of HHBSS. Labeled thalamic cells (8×10^4 cells/disk) in 90 μl of CM and, if applicable, fresh additives were then applied to slices. Cocultures were returned to the incubator for 3 hr, rinsed to remove nonattached cells, fixed, counterstained with bisbenzamide, and mounted as previously described.

Staining of Culture Slices for CS and Hyaluronan

Forebrain slices, treated as above, were rinsed, fixed (4% paraformaldehyde, 25 min), and washed into PBS. For CS-staining, rinses and dilutions were done with a blocking solution of 1% fish gelatin (Sigma) in PBS with 0.04% sodium azide. Tissue was blocked for 45 min, and left overnight at 4°C in either blocking solution or antibody (CS-56 monoclonal anti-CS [Sigma], 1:500). Slices were rinsed (4×10 min), gently agitated for 4 hr in secondary antibody (Cy3-conjugated, goat anti-mouse IgM, μ chain specific [Jackson Immunoresearch], 1:200), rinsed again (5×10 min), counterstained (10 μg/ml bisbenzamide in PBS), and mounted in saturated sucrose with 0.1% azide and an anti-bleaching agent (Prolong™ [Molecular Probes], component A).

For hyaluronan staining, rinses and dilutions were done with 3% BSA (Sigma) in 0.1 M Tris–HCl (pH 8), 150 mM NaCl. Tissue was blocked in this buffer plus 0.3% Triton X-100 and 0.1% sodium azide for 2 hr at room temperature, rinsed (4×10 min), and left overnight at 4°C in rinse solution or 1 μg/ml biotinylated hyaluronan-binding protein (Green et al., 1988). Slices were rinsed (4×10 min) and gently agitated for 2 hr in 1 μg/ml Texas red–conjugated avidin (Zymed). After rinsing (5×10 min), slices were counterstained and mounted as above.

Data Collection and Analysis

Slices were selected for quantitation based solely on whether cortical anatomy, as detected by bisbenzamide staining, was preserved. Cell attachment was measured as previously described, using a fluorescence microscope and the Neuron Tracing System (Eutectics Electronic Inc., Raleigh, NC). Switching between bisbenzamide and rhodamine filters allowed for simultaneous tracing of zone borders and attached thalamic cells. For cultures in which exogenous CS was used, measurements were made from images collected with a CCD camera and NIH image software.

Neurites were detected using fluorescence microscopy, and were only scored if their entire length, from origin to growth cone, could be seen. A calibrated reticule was used to determine if the origin of a neurite was within 25 μm of the subplate/cortical plate border.

Photos of CS immunostaining were taken on an epifluorescence microscope; however, a confocal microscope was used to quantitate fluorescence: gain and black levels were set so that all pixels in images of stained slices were below saturation and all pixels in control images (secondary antibody alone) were above zero. Data were collected from fields spanning the widths of the cortical plate and the subplate/intermediate zone (three fields/zone). A maximum projection image was created from a z-series (12×4.5 μm optical sections) that began at the focal plane of the tissue surface and ended 54 μm deep to the surface. From this image, immunofluorescence levels were defined as the area under the pixel histogram ("pixel total") for each summed image divided by the area of the field itself and, for each slice and zone, were averaged among the

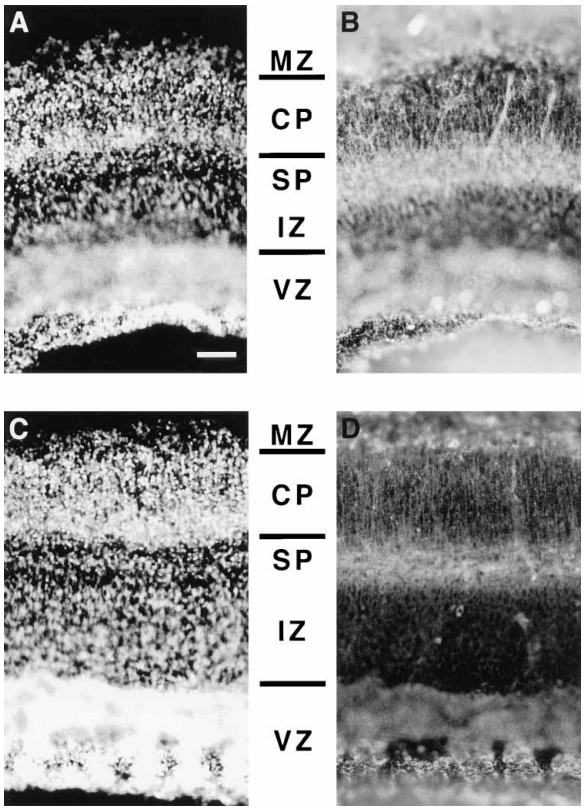

Figure 10. Treatment of E16 Sagittal Slices with Exogenous CS Does Not Alter Zone-Specific Immunolocalization of CS

Slices were cultured in the presence of 500 μg/ml CS (A and B) or carrier (complete medium, C and D) for 4 hr at 37°C, then rinsed, fixed, and immunostained as in Figure 4. Fields including the dorsomedial cortex are seen under bisbenzamide fluorescence (A and C) and antibody staining under rhodamine optics (B and D). (B) and (D) were photographed for identical exposure times. The results show that exposure to exogenous CS increases immunoreactivity throughout the slice—consistent with nonspecific binding or trapping of a small fraction of the applied CS—but it does not alter the zone-specific differences in CS immunoreactivity that are evident in an untreated slice. Bar, 50 μm.

three fields examined. For each zone, mean values from four slices are reported.

Attachment and immunofluorescence data were compared by a single factor analysis of variance (ANOV) or a two-tailed t test, as appropriate. A Newman-Keuls test verified outlier values. Neurite data were compared using a chi-squared test.

Microdissection and Proteoglycan Analysis

Vibratome slices of embryonic forebrain (200 μm) were further dissected under ice-cold 50 mM Tris–HCl (pH 8.0$^{4°C}$), 150 mM NaCl, 1 mM EDTA using tungsten microknives. Zones of the cerebral wall were discerned under a dissecting microscope (cf. Tuttle et al., 1995). To ensure that the cortical plate sample did not contain tissue from other zones, cuts were made so that the marginal zone and subplate/intermediate zone samples contained small amounts of cortical plate tissue. Tissue was collected into 3.5 ml of the above buffer plus 1 μg/ml Pepstatin, 1 mM PMSF, 250 μg/ml N-ethylmaleimide, and 1% CHAPS, and homogenized at 4°C in a teflon-on-glass homogenizer. Homogenates were centrifuged (423,500 × g, 40 min, 4°C), and supernatants collected, snap frozen, and stored at −80°C.

PGs were purified by anion exchange chromatography (Herndon and Lander, 1990). Extracts from several dissections were pooled (58 slices total) for each zone (total protein by amido black binding [Schaffner and Weissman, 1973]: marginal zone, ~34 μg; cortical plate, ~60 μg μg/ml; subplate/intermediate zone, ~54 μg; ventricular zone, ~64 μg). DEAE Spectra/Gel™ M beads (Spectrum), washed in homogenization buffer, were added to each of the four samples (~2 μl packed beads/μg total protein; ~40 ml total sample volumes), which were gently agitated overnight. Bead slurries were transferred to columns and washed sequentially (~7 ml/hr) with buffers C, D, E, and F of Herndon and Lander (1990), modified so that CHAPS (0.5%) replaced Triton X-100. Beads were then washed sequentially with 100 mM Tris–HCl (pH 8.0), 0.5% CHAPS (8 ml); 50 mM Tris–HCl (pH 8.0), 0.5% CHAPS (7 ml); and 50 mM Tris–HCl (pH 8.0), 150 mM NaCl (8 ml). Bound material was radioiodinated using chloramine T (Herndon and Lander, 1990) and eluted with 50 mM Tris–HCl (pH 8.0), 750 mM NaCl, 0.5% CHAPS. Samples were brought to ~300 μg/ml BSA (crystalline, ICN), snap frozen, and stored at −80°C.

For core protein analysis, PGs were diluted 4-fold in 50 mM Tris, 15 mM phosphoric acid (pH adjusted with HCl to 7.1$^{37°C}$), 1 mM EDTA, 1 μg/ml Pepstatin, 1 mM PMSF, 250 μg/ml N-ethylmaleimide, 400 μg/ml BSA, and digested with either Ch'ase (0.05 U/ml), a combination of Ch'ase and heparitinase (4 μg/ml; see Herndon and Lander, 1990), or no enzyme at 37°C for 3 hr. Digested samples were analyzed by SDS–PAGE under reducing conditions, and autoradiographed using HyperFilm™-MP (Amersham) at −80°C.

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